ARGUMENT

Claims 1-19 and 25-27 have been previously withdrawn from consideration. The Examiner in the current Office Action has withdrawn claims 22 and 23. Claims 20, 21 and 24 are currently pending in this application.

Rejection of Claim 20 under 35 U.S.C. § 103

The Examiner has rejected Claim 20 under 35 U.S.C. § 103 as being unpatentable over Sessler (U.S. Patent No. 5,622,946) in view of Lehninger (Biochemistry, 2nd Edition, pages 641-642, Worth Publishers (1975)). The Examiner has maintained his rejection of Claim 20 based on his position that since Claim 20 does permit ionizing radiation, it is not distinguished from Sessler.

Sessler teaches a method of radiation therapy, which requires administering ionizing radiation in the proximity of the neoplasm or atheroma for the compound to be effective. Applicants' respectfully submits that amended Claim 20 does not require/permit ionizing radiation and is thus distinguished over Sessler. Applicants respectfully submit that amended Claim 20 overcomes Examiner's instant rejection. Reconsideration and removal of the instant rejection is respectfully requested.

Claim 20 is also rejected by the Examiner under 35 U.S.C. § 103 as being unpatentable over Sessler (U.S. Patent No. 5,622,946) in view of Lehninger (Biochemistry, 2nd Edition, pages 503-504, Worth Publishers (1975)). As discussed above, Sessler teaches a method of radiation therapy, which requires administering ionizing radiation in the proximity of the neoplasm or atheroma for the compound to be effective. Applicants' respectfully submits that amended Claim 20 does not require/permit ionizing radiation. The Examiner also indicates that an individual can breathe oxygen and by doing so administers to himself (or herself) a "cellular metabolite." Applicants respectfully submit that, as discussed with the Examiner, the cellular metabolite used in the present invention is not oxygen. As indicated in the definitions section, page 18, lines 17-22 of the specification, Applicants have described the term cellular metabolite to have a standard biochemical reduction potential more negative than the standard biochemical reduction potential of oxygen/hydrogen peroxide. The present invention thus excludes oxygen as a cellular metabolite. The definition further provide examples of cellular metabolite to include NAD(P)H (i.e., NADPH and/or NADH), $FADH_2$, ascorbate, reduced glutathione, dihydrolipoic acid and the like. Applicants respectfully submit that amended Claim 20 and the above discussion overcomes the instant rejection of Claim 20. Reconsideration and removal of the instant rejection is respectfully requested.

Rejection of Claims 20 and 21 under 35 U.S.C. § 103

The Examiner has rejected Claims 20 and 21 under 35 U.S.C. § 103 as being unpatentable over Vogel (U.S. Patent No. 5,244,671). The Examiner has based his instant rejection on the disclosure by Vogel of the use of photoactivatable porphycenes and production of singlet oxygen following irradiation. Applicants respectfully assert that the Examiner's rejection based on Vogel is inappropriate for the following reasons.

Applicants respectfully submit that, as discussed with the Examiner, photoactivable porphycenes in Vogel are not the same as texaphyrins in the instant case. The porphycene compounds differ from texaphyrins used in the present case. Porphycene compounds in Vogel, unlike texaphyrins in the instant case, do not have a covalently bound metal atom in it. A description of porphycenes can be found in column 3 of the Vogel patent. Applicants respectfully submit that porphycenes are different from texaphyrins. Reconsideration and removal of the instant rejection of Claim 20 based on the dissimilar porphycenes is respectfully requested.

Also, as discussed in our response to the earlier Office Action, Vogel, in column 7, lines 26-29, indicate that they use ascorbic acid as an antioxidant to help maintain the chemical stability of a formulation containing porphycenes, thereby suggesting that ascorbic acid does not react with photoactivable porphycenes. Applicants respectfully submit that unlike photoactivable porphycenes, texaphyrins, and in particular motexafin gadolinium, react with ascorbate in neutral buffer to produce reactive oxygen species as reported by Magda et al., in Chem. Communication, pgs. 2730-2731 (2002) and Magda et al., in Int. J. Radiation Oncology Biol. Phys., vol. 51, pgs. 1025-1036 (2001) (copies of the preceding two articles are included for the Examiner's reference). Based on the findings in Magda et al., Applicants submit that production of reactive oxygen species cannot aid in maintaining chemical stability. Additionally, Vogel requires use of some form of irradiation while amended Claim 20 does not require any form of irradiation. Applicants respectfully submit that the above discussion points to the non-similarity between Vogel and the presently claimed invention and thereby overcomes the Examiner's instant rejection. Applicants submit that reconsideration and removal of the instant rejection is in order.

The Examiner has further rejected Claims 20 and 21 under 35 U.S.C. § 103 as being unpatentable over Vogel (U.S. Patent No. 5,244,671) in view of Kimoto (Cancer Research 43(2), 824-8 (1983) or Bram (Nature 284(5757) 629-31 (1980)). Applicants' submit that their above discussion of the reasons why rejection of the instant Claims 20 and 21 based on Vogel is improper applies in this rejection as well. In addition, Applicants submit that Kimoto and

Bram do not apply since they are different than the present invention. Kimoto clearly states that ascorbate in an aqueous solution is oxidized in the presence of cupric ion, thus producing reactive oxygen species and exhibiting cytotoxicity. Kimoto requires cupric ions while the present invention does not. Bram, on page 630, column 1, third paragraph, first line, indicates that they studied the inhibitory effects of copper as sulphate, gluconate and acetate salts. Both Kimoto and Bram require copper ions in contrast to the texaphyrin compounds used in the present invention which do not require, contain or generate copper ions. One skilled in the art would not be motivated to use texaphyrins, which are not a source of copper ions, based on the use of cupric ions and ascorbate to generate reactive oxygen. The present invention is thus easily distinguishable from both Kimoto and Bram. Applicants respectfully urge reconsideration and removal of the instant rejection of Claims 20 and 21 based on Vogel in view of Kimoto and Bram.

The Examiner has also rejected Claims 20 and 21 under 35 U.S.C. § 103 as being unpatentable over Platzek (U.S. Patent No. 6,136,841). Applicants respectfully submit that amended Claim 20 does not require any irradiation, photo dynamic or otherwise, while Platzek teaches porphyrins as suitable pharmaceutical agents for use in photodynamic therapy. The rejection based on Platzek is thus not applicable. Reconsideration and removal of the instant rejection of Claims 20 and 21 under 35 U.S.C. § 103 as being unpatentable over Platzek is respectfully requested.

The Examiner has also rejected Claims 20 and 21 under 35 U.S.C. § 103 as being unpatentable over Platzek (U.S. Patent No. 6,136,841) in view of Kimoto (Cancer Research 43(2), 824-8 (1983)) or Bram (Nature 284(5757), 629-31 (1980)). As discussed above, Applicants respectfully submit that amended Claim 20 does not require any irradiation, photodynamic or otherwise, while Platzek teaches use of 3,-8 deuteroporphyrin derivatives in photodynamic therapy. Platzek does not teach or suggest that one could use texaphyrins as anticancer agents in the absence of photoirradiation. Also, as discussed above, Kimoto and Bram require copper ions along with ascorbate see any cytotoxity. Neither reference, by itself or in combination, teaches the use of texaphyrins and ascorbic acid, in the absence of ionizing or other form of radiation, as anticancer agents. Applicants respectfully assert that reconsideration and removal of the instant rejection of Claims 20 and 21 is in order.

REMARKS

Applicants would like to thank the Examiner for taking the time to discuss the instant Office Action with Applicants' undersigned Attorney. Applicants appreciate the Examiners

willingness, in response to the undersigned Attorney's suggestion, to contact Applicants undersigned Attorney after reviewing the instant response, should the Examiner continue to maintain any of the above-discussed rejections. Applicants believe that amended Claim 20 does address and overcome all the rejections in the instant case. Applicants believe that the present response is fully responsive to the Office Action issued by the Examiner in this case.

Applicants submit that the instant amendment to the claims and the specification does not introduce any new matter. Amendment to the specification deals with a typographical error on page 18, line 26, wherein the reduction potential for oxygen and hydrogen peroxide was incorrectly stated to be 0.273 mV when it should have been 0.273 V. The above error was inadvertent and unintentional and rectifying this error does not add any new matter.

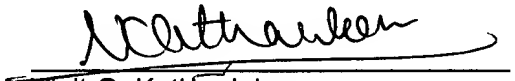
Applicants request a three-month extension for filing this response. Should the Examiner determine otherwise, the Examiner is hereby authorized to deduct any additional fees, if necessary, or credit any overpayment, to the Applicants Deposit Account No. 16-1450. Applicants respectfully request the Examiner to contact Applicants' undersigned Attorney should the Examiner have any questions.

Conclusion

For the foregoing reasons, Applicant believes all the pending claims are in condition for allowance and should be passed to issue. If the Examiner feels that a telephone conference would in any way expedite the prosecution of the application, please do not hesitate to call the undersigned at 408.774.0330.

Respectfully Submitted,

Date: 12th March '04



Vinit G. Kathardekar
Reg. No. 39,461

Pharmacyclics, Inc.
995 E. Arques Avenue
Sunnyvale, CA 94085
Phone: 408.774.0330
Fax: 408.774.0340
Email: Legal@pcyc.com

COMMUNICATION

ChemComm www.rsc.org/chemcomm

Motexafin gadolinium reacts with ascorbate to produce reactive oxygen species†

Darren Magda,*[a] Nikolay Gerasimchuk,[a] Philip Lecane,[a] Richard A. Miller,[a] John E. Biaglow[b] and Jonathan L. Sessler[c]

[a] *Pharmacyclics, Inc., 995 E. Arques Avenue, Sunnyvale, CA 94085, USA. E-mail: dmagda@pcyc.com; Fax: 408 774 0340; Tel: 408-774-0330*

[b] *Department of Radiation Oncology, University of Pennsylvania, Philadelphia, PA 19104*

[c] *Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, TX 78712*

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Motexafin gadolinium (MGd) oxidizes ascorbate, in neutral buffer and in cell culture, forming reactive oxygen species and a coordination polymer with oxalate.

An important function of metabolism is to maintain cellular systems in a reduced state relative to the aerobic environment. Although reducing metabolites such as glutathione, NADPH, and ascorbate are central to cellular antioxidant defense, these compounds have reduction potentials sufficiently negative for spontaneous electron transfer to oxygen to occur in the presence of an appropriate catalyst. Such catalysts, termed 'redox mediators', have provided important tools with which to examine the impact of oxidative stress in living systems, including its effect in the biological response to ionizing radiation.[1]

The gadolinium(III) complex **1** (motexafin gadolinium, Xcytrin®, MGd) is currently in advanced stage clinical development as an adjuvant to radiation therapy.[2] MGd has previously been reported to enhance the efficacy of radiation in animal tumor models.[3] Recent studies *in vitro*, however, have revealed a surprising sensitivity to the choice of medium.[4] In particular, it was discovered that the amount of ascorbate present correlated with the cytotoxicity of the drug. This finding has led us to consider that MGd can function as a redox mediator. The generation of reactive oxygen species, if occurring, could provide a basis for the mechanism of biological action of this agent. Support for this hypothesis is presented below.

The oxidation of ascorbate in a neutral pH saline solution was quantified by monitoring the decrease of its absorbance at 266 nm.[5] Addition of a catalytic amount of MGd to a solution of ascorbate resulted in no spectral changes in solutions purged with argon. However, under ambient atmospheric conditions, a rapid decrease of the ascorbate absorbance was observed, the rate of which was initially linear and which decreased to background level within *ca.* 30 minutes (Fig. 1A). During the course of *ca.* 60 minutes, the Soret and Q-like absorption bands of the complex at 470 nm and 742 nm, respectively, were converted with clean isosbestic points to new absorbances at 510 nm and 780 nm.

Spectra obtained under an oxygen atmosphere displayed similar features (Fig. 1B), with the exception that the oxidation of ascorbate proceeded with *ca.* 3-fold greater initial rate and to a much greater degree of completion (*Cf.*, also, Supplementary Information†). Under ambient conditions, addition of catalase had no significant effect on the rate of ascorbate or oxygen consumption (Table 1).‡ It did, however, lead to a two-fold decrease in the rate at which the species absorbing at 510 and 780 nm, referred to as **2**, was formed. Superoxide dismutase slowed the rate of ascorbate and oxygen consumption by a factor of two, and, moreover, slowed formation of the species absorbing at 510 and 780 nm by at least ten-fold (Table 1).

The identity of the new species **2** was investigated. It was observed to precipitate upon prolonged incubation in buffer, and the resulting solid was catalytically active when added to fresh ascorbate solution. Addition of methanol to this solid resulted in gradual regeneration of the starting complex. Based on these observations, a range of standard chemical analyses, and the finding that identical spectral changes occur upon incubation of complex **1** in buffer with either dehydroascorbate (slow) or disodium oxalate (rapid, *Cf.*, Supplementary Information†),§ we propose that species **2** consists of a coordination polymer with oxalate as shown in Fig. 2. Oxalate is a known decomposition product of dehydroascorbate, which is unstable at physiologic pH and in the presence of superoxide.[6]

The effects of oxygen and enzymes outlined above are consistent with the existence of an equilibrium between the single-electron reduced form of MGd and oxygen on the one hand and superoxide anion and MGd **1** on the other (Eqn. 1).¶ [7,8] In accordance with this scheme, perturbations decreasing the

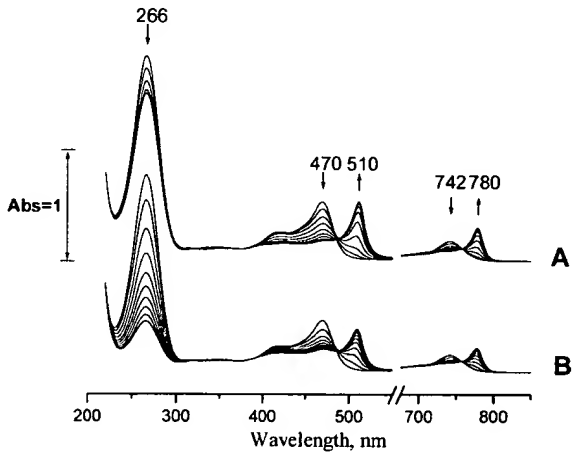

Fig. 1 Spectral changes occurring upon incubation of MGd with ascorbate in buffered solution. A solution of ascorbic acid (1.25 mM) in 50 mM HEPES buffer, pH 7.5, 100 mM NaCl (Chelex-100™ -treated, all concentrations final) was placed in a 1 mm quartz cuvette. The UV-visible spectrum was recorded for 60 minutes following addition of MGd (62 μM). A. Ambient conditions. B. Oxygen atmosphere.

Table 1 Rates of oxygen, ascorbate, and MGd decrease[a]

	$\delta[O_2]/\delta t$	$\delta[Asc]/\delta t$	$\delta[MGd]/\delta t$
No enzyme	5.56 ± 1.13	9.05 ± 0.64	1.22 ± 0.40
Catalase[b]	4.63 ± 0.80	8.30 ± 0.48	0.69 ± 0.07
SOD[c]	1.93 ± 0.15	4.48 ± 0.45	0.09 ± 0.04
Catalase + SOD[bc]	2.38 ± 0.30	3.94 ± 0.34	0.10 ± 0.09

[a] Reaction of ascorbate (1.2 mM) with MGd (62 μM) in 100 mM NaCl, 50 mM HEPES buffer, pH 7.5. Units are μM min^{-1}, mean ± std. dev. of 3 runs. [b] Catalase [EC 1.11.1.6], 2600 units mL^{-1}, or [c] superoxide dismutase [EC 1.15.1.1], 100 units mL^{-1}, added prior to MGd.

† Electronic supplementary information (ESI) available: experimental description and results. See http://www.rsc.org/suppdata/cc/b2/b208760j/

DOI: 10.1039/b208760j

concentration of reduced MGd, such as an increase in oxygen, would increase the rate of ascorbate oxidation by regeneration of **1**. Superoxide dismutase, on the other hand, would decrease the rate of ascorbate oxidation by inhibiting reduction of superoxide by ascorbate.[5] Removal of superoxide, whether by reduction or disproportionation, would lead to complex regeneration and formation of hydrogen peroxide and dehydroascorbate.‖[9]

$$MGd + O_2^{\cdot -} \rightleftharpoons MGd^{\cdot -} + O_2 \quad (1)$$

As a specific test as to whether redox cycling by MGd occurs *in vitro*, A549 (human lung cancer line) cells** were incubated with MGd, MGd and ascorbate, or pre-formed oxalate complex **2**. An inhibitor of glutathione synthesis, L-buthionine-[*S*,*R*]-sulfoximine (BSO), was also added, to inhibit hydrogen peroxide removal by glutathione peroxidase. After washing, cultures were treated with dichlorofluorescin acetate (DCFA). Cell suspensions were analyzed by flow cytometry, using an excitation wavelength of 488 nm, and emission filters at 530 nm (FL1) and >650 nm (FL3). As shown in Fig. 3, an increase in FL1 fluorescence was observed in cells treated with MGd (or **2**) and BSO, indicative of DCFA oxidation to form dichlorofluorecein (DCF).[10] This FL1 signal was increased by co-incubation with ascorbate and also in cells incubated with pre-formed **2**. Moreover, this latter increase correlated with that in the FL3 channel, derived from the fluorescence of MGd at 760 nm. This finding leads us to suggest that the cellular uptake of MGd is facilitated in the presence of ascorbate *via* intermediate formation of the oxalate complex **2**.[11]††

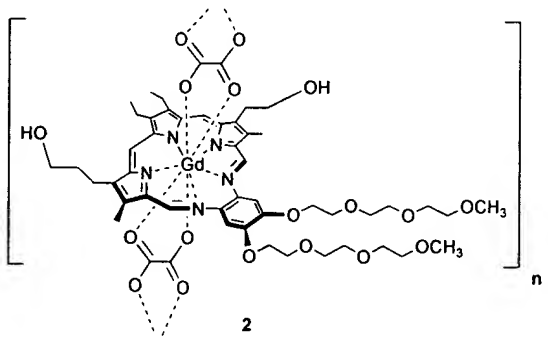

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Fig. 2 Structure proposed for motexafin gadolinium oxalate complex **2**, formed from the corresponding monomeric diacetate complex **1**.

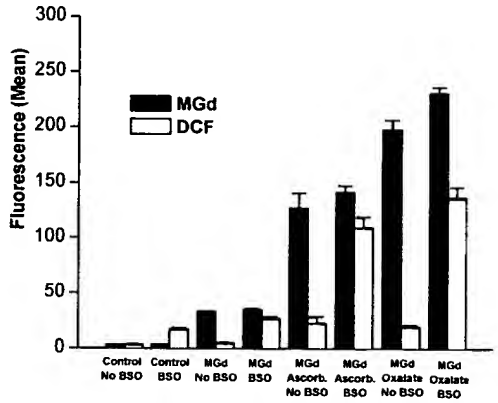

Fig. 3 Analysis of A549 lung cancer cells by flow cytometry. A549 cells (4×10^5 cells) were treated with MGd (50 μM) with or without ascorbate (100 μM) or MGd oxalate complex **2** (50 μM) in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum for 22 h. BSO (100 μM) was also added where indicated. Cultures were washed with Dulbecco's phosphate buffered saline (PBS), and treated with DCFA (0.25 μg mL^{-1}) in Hank's sterile saline for 10 minutes. Cultures were washed with PBS, treated with trypsin, and the resulting suspensions subjected to analysis by flow cytometry. Mean fluorescence at 530 nm (DCF) and >650 nm (MGd) is shown. Ascorbate treatment alone had no effect on fluorescence (see Supplementary Information†). Error bars indicate standard deviation (n = 3).

The above experiments, considered in concert, provide support for the proposal that reactive oxygen species are formed intracellularly in the presence of MGd, especially in ascorbate-containing media. Reactive oxygen species generation could contribute to the increased radiation response observed in preclinical models.[3] *tert*-Butyl hydroperoxide, for example, has been shown to enhance radiation response *in vitro*.[12] Interestingly, texaphyrins can display considerable (as much as 10-fold) selective accumulation in tumor tissue.[2,3] To our knowledge, the development of a redox cycling agent which localizes selectively to neoplasia *in vivo* would represent a new approach to enhancing the therapeutic response to ionizing radiation or chemotherapy.[13] Studies to evaluate the consequences of electron transfer to MGd from ascorbate and other reducing metabolites (*e.g.*, NADPH) present in biological systems are currently underway.

Notes and references

‡ Oxygen was measured using a ruthenium bipyridine tipped fiber optic probe (Ocean Optics, Inc.) calibrated with a Clark electrode.

§ Presence of oxalate in compound **2** was confirmed using ^{13}C NMR, IR, and ion chromatographic analyses (Supplementary Information†).

¶ The equilibrium proposed was examined recently using pulse radiolytic techniques.[7] The equilibrium constant was reported to be *ca.* 3 in favor of reduced MGd, underscoring the fact that MGd is more electron affinic than oxygen.[8]

‖ Non-catalyzed superoxide disproportionation is estimated to occur with a rate constant of 1×10^5 $M^{-1}s^{-1}$ at pH 7.4.[9]

** Human lung cancer cell line A549 was obtained from the American Type Culture Collection.

†† Complex **2** was found to be significantly less fluorescent than MGd[11] The FL3 signal observed in cells treated with complex **2** may derive in part from intracellular re-formation of MGd.

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Int. J. Radiation Oncology Biol. Phys., Vol. 51, No. 4, pp. 1025–1036, 2001

0360-3016/01/$–see front matter

ELSEVIER

PII S0360-3016(01)01810-7

BIOLOGY CONTRIBUTION

REDOX CYCLING BY MOTEXAFIN GADOLINIUM ENHANCES CELLULAR RESPONSE TO IONIZING RADIATION BY FORMING REACTIVE OXYGEN SPECIES

DARREN MAGDA, PH.D.,* CHERYL LEPP, M.S.,* NIKOLAY GERASIMCHUK, PH.D.,* INTAE LEE, PH.D.,* JONATHAN L. SESSLER, PH.D.,† ALICE LIN, M.S.,* JOHN E. BIAGLOW, PH.D.,‡ AND RICHARD A. MILLER, M.D.*

*Pharmacyclics, Inc., Sunnyvale, CA; †University of Texas at Austin, Austin, TX; ‡University of Pennsylvania, Philadelphia, PA

Purpose: To examine the mechanism of radiation enhancement by motexafin gadolinium (Gd-Tex) *in vitro*.
Methods and Materials: Oxidation of ascorbate and NADPH by Gd-Tex was evaluated in a neutral buffer. Growth inhibition of human uterine cancer cell line MES-SA was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye. Clonogenic assays were used to measure radiation response in MES-SA, A549 human lung carcinoma, E89, a CHO cell line variant deficient in glucose-6-phosphate dehydrogenase activity, and murine lymphoma cell lines LYAR and LYAS.
Results: Gd-Tex catalyzed the oxidation of NADPH and ascorbate under aerobic conditions, forming hydrogen peroxide. Decreased viability was observed in MES-SA cells incubated with Gd-Tex in media containing NADPH or ascorbate. Gd-Tex and ascorbate increased fluorescence in dichlorofluorescin acetate–treated cultures. Synergistic effects on the aerobic radiation response in MES-SA and A549 were seen using Gd-Tex in combination with L-buthionine-(S,R)-sulfoximine (BSO). Incubation with Gd-Tex in the presence of ascorbate increased the aerobic radiation response of E89 and the apoptosis-sensitive B-cell line (LYAS).
Conclusions: Gd-Tex sensitizes cells to ionizing radiation by increasing oxidative stress as a consequence of futile redox cycling. Optimization of the concentration of ascorbate (or other reducing species) may be required when evaluating Gd-Tex activity *in vitro*. © 2001 Elsevier Science Inc.

Texaphyrin, Radiation, Sensitizer, Ascorbate, Superoxide.

INTRODUCTION

Compounds that display affinity for electrons can potentiate the biologic effect of ionizing radiation (1). Both indirect and direct mechanisms of action have been proposed: Sensitization may occur indirectly by altering levels of radioprotective metabolites (e.g., glutathione), or the sensitizer may interact directly with cellular macromolecules to cause or enhance damage. Examples of radiation sensitizers believed to act by the former mechanism include "diamide" (diazenedicarboxylic acid *bis*[*N*, *N*′-dimethylamide]), *tert*-butyl hydroperoxide, L-buthionine-(S,R)-sulfoximine (BSO), and other thiol depleters (2–6). On the other hand, tirapazamine, nitroimidazoles, and oxygen react directly with DNA or other biologic targets (7–9). In general, "oxygen mimetic" sensitizers of this latter group are most effective under hypoxic conditions, where activity is not masked by the competitive activity of oxygen.

Solvated electrons produced by the radiolysis of water may serve to reduce the electron-affinic sensitizer (1). However, even in the absence of ionizing radiation, electron transfer to a radiation sensitizer may occur in the presence of cellular metabolites that have a more negative standard reduction potential, e.g., NADPH, reduced glutathione, flavins, or ascorbate (10, 11). This process consumes the reducing metabolite, which must then be replenished. Moreover, in the presence of oxygen, further electron transfer can form reduced oxygen species (e.g., superoxide and hydrogen peroxide) and regenerate the sensitizer. Overall, such "redox cycling" can lead to a condition of oxidative stress.

Texaphyrins are porphyrin-like macrocycles that form highly stable complexes with large metal cations (12). Motexafin gadolinium (Xcytrin, Gd-Tex, Fig. 1) has been reported to enhance the efficacy of radiation in animal tumor models and is currently in Phase III clinical development as an adjuvant to radiation therapy (13–17). Gd-Tex is electron affinic, with a first reduction potential near −50 mV (NHE) (18). To better understand the mechanism of its action as a radiation enhancer, the properties of the complex were examined under *in vitro* conditions. Our findings lead us to suggest that Gd-Tex sensitizes cells through a novel mechanism of action, wherein oxidative stress caused by redox cycling leads to an enhanced radiation response.

Reprint requests to: Darren Magda, Ph.D., Pharmacyclics, Inc., 995 E. Arques Avenue, Sunnyvale, CA 94085.

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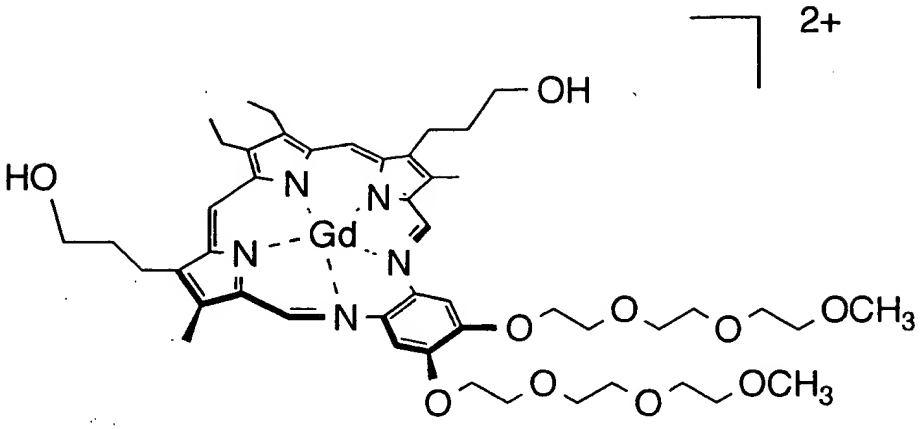

Fig. 1. Structure of motexafin gadolinium (Gd-Tex). Axially coordinated counterions (e.g., acetate) are omitted for clarity.

METHODS AND MATERIALS

Cell lines and materials

Reagents were purchased from Sigma Chemicals, unless otherwise indicated. The diacetate form of motexafin gadolinium was prepared as previously described (18).

Human uterine cancer cells MES-SA were provided by B. Sikic (Stanford School of Medicine) (19). The derivation and characterization of Chinese hamster ovary cell lines E89 and K1 have been described previously (20). Murine B-lymphocytes LYAR and LYAS were provided by D. Voehringer (Stanford School of Medicine) (21). Human lung cancer line A549 was obtained from the American Type Culture Collection.

Oxidation of NADPH

Cofactor oxidation was monitored by ion exchange high-performance liquid chromatography (HPLC) using a Nucleogel DEAE 60-7, 125 × 4-mm column. In brief, a gradient was prepared using 8 mM and 750 mM potassium phosphate, pH 3.8, mobile phase solutions to achieve baseline resolution of reduced and oxidized cofactors. Detector wavelength was 260 nm, and column temperature was 40°C. A solution of Gd-Tex in American Chemical Society (ACS) grade water (Aldrich Chemical), e.g., 0.05 molar equivalent, was added to a solution of NADPH in 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) buffer, pH 7.5, 100 mM NaCl (all concentrations final), vortexed briefly, and placed in the HPLC sampler for immediate injection. Further injections were made at appropriate intervals, whereupon the peak area of both NADPH and $NADP^+$ were measured and used to calculate the extent of reaction at a given time point. This process was repeated at various concentrations of NADPH. Initial rates (measured in triplicate) were used to derive a saturation curve, which was fitted to the Michaelis-Menten equation using KaleidaGraph software (Synergy Software, Reading, PA), i.e., initial reaction velocity/catalyst concentration = (k_{cat}) (NADPH concentration)/$(K_M$ + NADPH concentration), where k_{cat} is the first-order rate constant, and K_M is the dissociation (or Michaelis) constant for the catalyst/substrate complex.

Hydrogen peroxide assay

Hydrogen peroxide was measured using a Bioxytech H_2O_2-560 quantitative assay kit obtained from R&D Systems (Minneapolis, MN). In brief, a reaction mixture was prepared containing substrate, buffer, and water. A lesser amount of texaphyrin complex, e.g., 0.05 molar equivalent, was added to this reaction mixture, which was vortexed and incubated at ambient temperature in the dark. Aliquots of 50 μL were removed every 20 min and added to a colorimetric reagent sensitive to the presence of H_2O_2. Ascorbate-containing solutions were analyzed by visible spectroscopy 25–30 min after addition to the colorimetric reagent, to minimize the effect of the slow background oxidation of this substrate. A 25-mM solution of H_2O_2 was prepared by dilution of 30% H_2O_2. The absorbance at 240 nm was used to standardize this solution, which was further diluted and used to construct a standard H_2O_2 curve at 560 nm in conjunction with the colorimetric reagent. Further details on the use of the Bioxytech H_2O_2-560 kit are available from the package insert. Catalase [EC 1.11.1.6] (260,000 u/mL) was obtained from Roche Molecular Biochemicals (Indianapolis, IN).

Ascorbate oxidation

The kinetics of ascorbate oxidation by Gd-Tex were measured by following the decrease in absorbance at 266 nm (22). A solution of Gd-Tex in ACS grade water (Aldrich Chemical) was added to a solution of ascorbic acid in 50 mM HEPES (Gibco BRL, Rockville, MD) buffer, pH 7.5, 100 mM NaCl (all concentrations final). The buffer was treated with Chelex 100 (Bio-Rad, Hercules, CA) to remove transition metal cation contaminants before use. Sodium chloride (99.999%) and NaOH (semiconductor grade) were from Aldrich. Initial rates were used to calculate a saturation

curve, as described above with NADPH. Data from the first 40 min of reaction were used in the initial rate calculations. Concentrations were chosen such that the measured rates reflect multiple turnover conditions. Menadione (Aldrich Chemical) was freshly prepared as a 0.5-mM stock solution in dimethylsulfoxide (DMSO).

Cell proliferation studies

The proliferation of MES-SA human uterine cancer cells in the presence of substrate or Gd-Tex was used to assess the formation of reactive oxygen species under cell culture conditions. MES-SA cells were allowed to adhere to 96-well microtiter plates (4,000 cells per well) overnight in 200 μL RPMI 1640 medium containing 10% dialyzed fetal bovine serum (FBS, Gibco-BRL) and 2% penicillin/streptomycin solution (Sigma). A freshly prepared stock solution of ascorbic acid or NADPH, 3.0 mM in medium (100 μL), was serially diluted (1:3) down the plate; the final 100 μL was discarded. Medium (20 μL) was removed from all wells, whereupon stock solutions of Gd-Tex (1 mM, 500 μM, 250 μM, or 125 μM diluted in medium and 5% mannitol) were added to the plates to give a final volume of 200 μL. Final mannitol concentration was 0.25% in all wells. The plates were incubated at 37°C under a 5% CO_2/95% air atmosphere. Gd-Tex–containing medium was exchanged for fresh medium (180 μL) after 5 h, and the plates were incubated an additional 72 h before analysis for viability using the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (23). Plate absorbances were normalized to wells containing neither ascorbate nor complex to allow plate-to-plate comparison. In control experiments, catalase [EC 1.11.1.6] and superoxide dismutase [EC 1.15.1.1] (Roche Molecular Biochemicals, 52 U and 80 U, respectively) were present in all wells before addition of Gd-Tex.

Fluorescence assay for reactive oxygen species

Dichlorofluorescein fluorescence was measured in cell cultures treated with Gd-Tex and ascorbate (24). MES-SA cells were plated in 96-well microtiter plates at 1×10^4 cells/well and allowed to adhere for ~36 h. A stock solution of 3 mM ascorbate was diluted serially. Stock solutions of Gd-Tex were added as above, and the plates were incubated at 37°C for 4 h. Gd-Tex–containing medium was removed, and the cells were washed with fresh medium and phosphate-buffered saline (PBS, supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM KCl, and 20 mM glucose); then a solution of dichlorofluorescin acetate (DCFA, 5 μg/mL) in PBS was added. The fluorescence (arbitrary units) was measured using a Fluoroskan Ascent FL instrument (Labsystems, Helsinki, Finland) after incubation overnight at 37°C (excitation wavelength 485 nm, emission wavelength 538 nm).

Clonogenic assays

The response of MES-SA cells to ionizing radiation was studied using a clonogenic assay. MES-SA cells (200–5,000 cells per flask) were plated in T-25 flasks in 8.5 mL McCoys 5A containing 10% FBS (Gibco-BRL) and 2% penicillin/streptomycin solution (Sigma) and incubated at 37°C overnight. Stock BSO, ascorbate, and Gd-Tex (or 5% mannitol vehicle) solutions were prepared, and 0.5 mL of each was added to each flask to give a final volume of 10 mL (Final concentrations of BSO, ascorbate, and Gd-Tex were 100 μM, 20 μM, and 50 μM, respectively), and the cells were incubated for 24 h, whereupon the flasks were irradiated using a ^{137}Cs irradiator (Model 40 Gammacell, J.L. Shepherd & Assoc., San Fernando, CA) at a dose rate of 0.805 Gy/min. Under these conditions, BSO lowered total glutathione levels to approximately 30% that of controls, regardless of treatment with Gd-Tex. Medium was removed immediately after irradiation, the cells were washed with fresh medium (5.0 mL), fresh medium (10 mL) was added, and the solution was incubated for an additional 11 days at 37°C. Cultures were fixed with isopropyl alcohol, stained with 1% crystal violet, and counted. Colonies comprising >50 cells were scored as positive. (Note: An attempt to use an alternate protocol wherein cells were detached using trypsin after Gd-Tex treatment was hampered by inhibition of this enzyme.)

The radiation response of the murine B-cell lymphoma sublines LYAR (apoptosis resistant) and LYAS (apoptosis sensitive) was measured by clonogenic assay. In this experiment, a suspension of 5×10^5 cells/mL was treated with Gd-Tex (50 μM) or 5% mannitol vehicle in RPMI 1640 medium supplemented with 10% FBS, glutamine (2 mM), antibiotics, and ascorbate (10 μM) for 17 h before irradiation. Subsequent to irradiation, as above, cells were pelleted, washed with medium, counted, resuspended in fresh medium (0.5 mL) at appropriate cell number, and added to methylcellulose (Methocult M3234, Stem Cell Technologies, Vancouver, BC, Canada; 3 mL). After a 7-day incubation at 37°C in 6-well plates (1 mL/well), colonies were counted against a black field using a stereomicroscope.

The radiation response of CHO cell variant E89 was measured as above with the following changes: Aliquots of 5×10^4 cells in 7 mL McCoys 5A medium (supplemented with 10% FBS, 25 mM HEPES buffer, and antibiotics) were added to T-25 flasks and allowed to grow for 3 days. At this time, medium was exchanged for freshly completed medium, and ascorbate (0 or 37.5 μM) and Gd-Tex (25 μM) or 5% mannitol vehicle was added. Flasks were irradiated as above, after a 3-h incubation at 37°C, and cells were washed with fresh medium and Hanks' solution. Cells were prepared for subculture using 0.25% trypsin (no EDTA) for 5 min. After centrifugation, resuspended cells were counted, plated in T-25 flasks, and incubated at 37°C for 8 days.

The radiation response of A549 was measured as above with the following changes: Aliquots of 2×10^4 cells in 7 mL RPMI 1640 medium (supplemented with 15% FBS, 25 mM HEPES buffer, and antibiotics) were added to T-25 flasks and allowed to grow for 5 days. At this time, medium was exchanged for freshly completed medium supplemented with ascorbate (15 μM), and Gd-Tex (50 μM) or

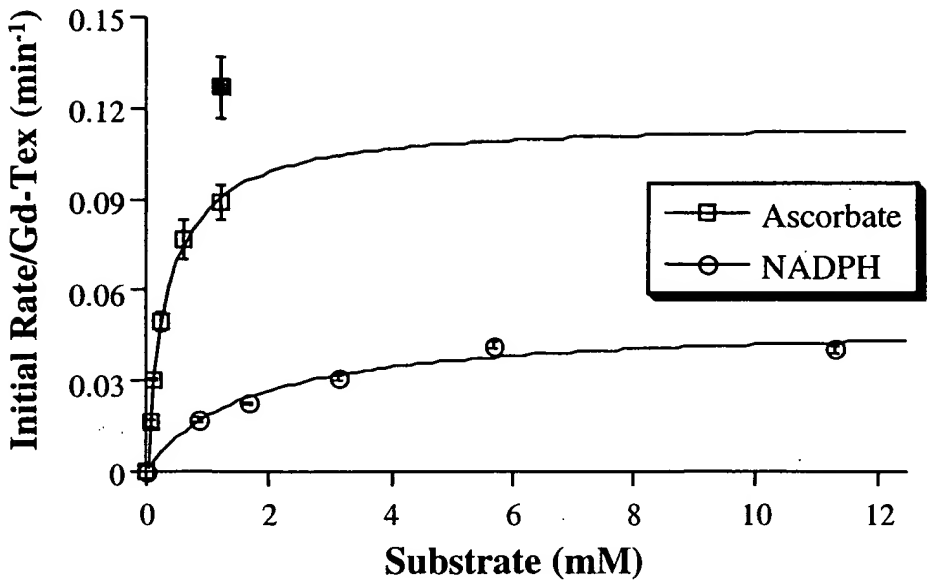

Fig. 2. Saturation curves for oxidation of ascorbate and NADPH by Gd-Tex in buffer (50 mM HEPES, pH 7.5, 100 mM NaCl). Initial rates of oxidation were measured by monitoring the decrease in ascorbate absorbance at 266 nm or HPLC peak area of reduced cofactor. Background rates of ascorbate autoxidation were subtracted. The rate of oxidation of 1.25 mM ascorbate by menadione (closed square) is shown for comparison. Error bars indicate standard error (n = 3).

BSO (100 μM) was added. Flasks were irradiated as above, after a 24-h incubation at 37°C. Cells were washed with fresh medium and Hanks' solution and prepared for subculture using 0.05% trypsin with 0.53 mM EDTA (Gibco/BRL) for 8 min. After centrifugation, resuspended cells were counted, plated in T-25 flasks, and incubated at 37°C for 8 days.

Survival curves were prepared by fitting the data to a linear-quadratic model ($SF = \exp(-\alpha D - \beta D^2)$, where SF is the surviving fraction, and D is the dose). Parameters were estimated using a nonlinear-weighted least-squares regression using the Excel program.

Thiol measurements

Reduced thiol measurements were performed using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) as previously described (20). The medium was exchanged on plateau-phase cultures of E89 grown in T-25 flasks 24 h before treatment with Gd-Tex (50 μM) or ascorbate (3.75 mM). After incubation for 3 h, the cultures were washed 3 times with Dulbecco's PBS, supplemented with $MgCl_2$ hexahydrate and $CaCl_2$ (0.1 g/L). Proteins were precipitated and cells lysed by treatment with 2 mL of a solution of 7% sulfosalicylic acid and EDTA (100 μM). An aliquot (1 mL) of supernatant was combined with 2 mL of a solution of 1.5 mM DTNB in 1 M potassium phosphate buffer, pH 7.4. Insoluble proteins were washed twice with 7% sulfosalicylic acid solution, then resolubilized in a 5-mL solution of 1.5 mM DTNB in 100 mM potassium phosphate buffer, pH 7.4 for 15 min at 37°C. The thiol concentrations were quantified by UV-vis spectroscopy using an extinction coefficient of 13,600 for reduced DTNB at 412 nm. Total protein levels in parallel cultures were measured using a commercial kit and following the manufacturer's protocol (BCA Protein Assay, Pierce, Rockford, IL).

RESULTS

Oxidation of NADPH by Gd-Tex

Co-incubation of NADPH with Gd-Tex converted the cofactor to its oxidized form, at catalytic concentrations of complex. The initial rate of oxidation correlated with cofactor concentration; i.e., the reaction displayed saturation kinetics (Fig. 2). Values of k_{cat} and K_M are compiled in Table 1.

The above studies were conducted under aerobic conditions. Carrying out analogous studies under an inert atmosphere led to rapid complex degradation, as evidenced by bleaching of the characteristic Gd-Tex UV-vis absorbance spectrum (data not shown). This observation is consistent with the idea that oxygen is serving as the ultimate electron acceptor under aerobic conditions, as has been observed with other electron-mediating com-

Table 1. Substrate oxidation data: Values obtained from HPLC (NADPH) or UV-vis (ascorbate) analysis of solutions of Gd-Tex and substrate in buffer (50 mM HEPES, 100 mM NaCl, pH 7.5)

	k_{cat} (min^{-1})*	K_M(mM)*
Gd-Tex/NADPH	0.049 ± 0.003	1.69 ± 0.17
Gd-Tex/ascorbate	0.125 ± 0.23	0.422 ± 0.211

* Average values from 3 independent determinations (± standard deviation).

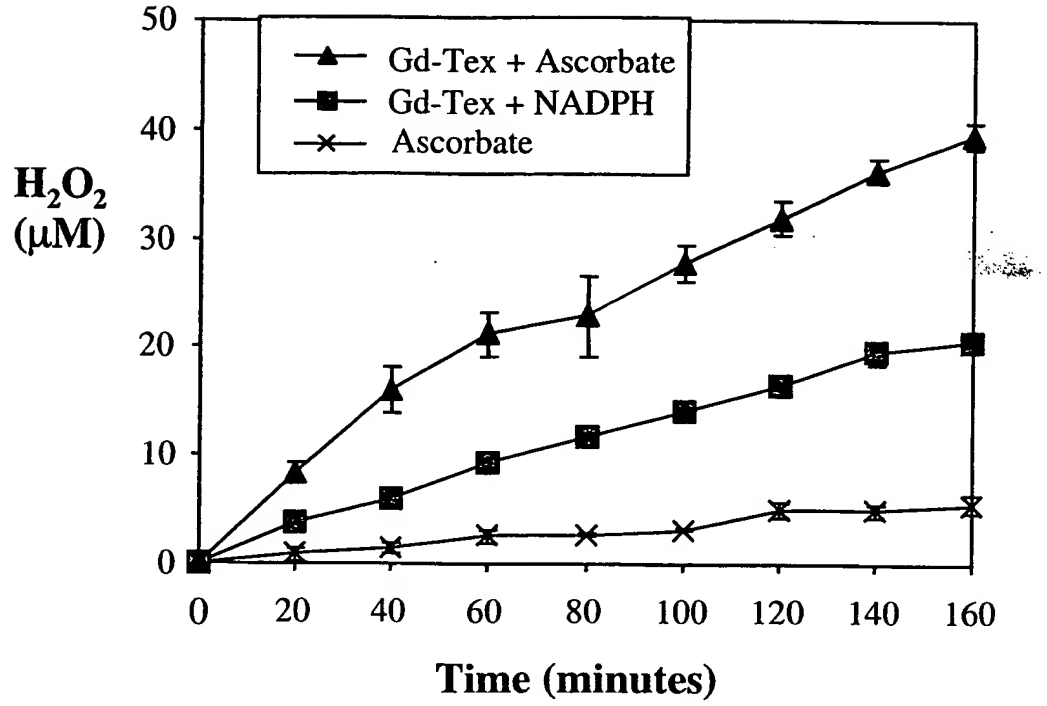

Fig. 3. Colorimetric determination of hydrogen peroxide formation in buffered solutions of NADPH or ascorbic acid. Solutions of NADPH or ascorbic acid (250 μM in buffer, as above) and Gd-Tex (12.5 μM, i.e., 0.05 molar equivalent in water) were mixed and incubated at ambient temperature. Error bars indicate standard deviation (n = 3). Background signal caused by oxidation of ascorbate by reagent has been subtracted.

pounds (25). The formation of hydrogen peroxide upon incubation of NADPH with Gd-Tex was measured using a colorimetric assay (Fig. 3). The rate of peroxide formation paralleled the rate of cofactor oxidation under the same conditions. Addition of catalase to the reaction mixture confirmed that the assay signal was due to hydrogen peroxide (data not shown).

Oxidation of ascorbate by Gd-Tex

Hydrogen peroxide analysis was also used to examine other biologic reductants as potential substrates for texaphyrin-based oxidation. Hydrogen peroxide was formed when ascorbic acid was substituted for NADPH in the reaction mixture (Fig. 3). Because of background oxidation of ascorbate in the peroxide assay, however, UV-vis spectroscopy was chosen to monitor the reaction rate more closely. The decrease in the ascorbate absorbance at 266 nm was measured as a function of time and used to calculate initial rates (22). Saturation kinetics were observed, as was the case when NADPH was chosen as the reductant, provided that concentrations of the Gd-Tex catalyst were kept low (Fig. 2). At high concentrations of both ascorbate and Gd-Tex, addition of superoxide dismutase lowered the measured rate. We interpret this as evidence of a competing process involving the reduction of superoxide by ascorbate (22). Catalytic constants for the oxidation of ascorbate by Gd-Tex are compiled in Table 1. This activity of Gd-Tex was compared with that of menadione, a water-soluble quinone often used to generate oxidative stress in cellular systems (25). In the presence of a catalytic amount of menadione, ascorbate oxidation was found to proceed at a rate similar to that observed using Gd-Tex (Fig. 2). NADPH, on the other hand, was not converted into its oxidized form by menadione under these conditions.

Cytotoxicity of Gd-Tex in cell culture

The proliferation of human ovarian cancer cell line MES-SA was used to assess the degree of ascorbate and cofactor oxidation under cell culture conditions. RPMI 1640 with dialyzed serum was chosen as the medium in these experiments to ensure the absence of adventitious ascorbate. As seen in Fig. 4a, a 5-h co-incubation of ascorbate and Gd-Tex resulted in decreased cell survival, as measured by tetrazolium salt (MTT) reduction. This effect was obviated partially by addition of catalase and superoxide dismutase (Fig. 4b). Gd-Tex showed an effect at lower concentrations of ascorbate (e.g., 37 μM) than NADPH (e.g., 333 μM, Fig. 4c); this is consistent with the respective values for the dissociation constants as listed in Table 1.

Fluorescence assay for reactive oxygen species

As a specific test for intracellular formation of reactive oxygen species, MES-SA cells were incubated with Gd-Tex for 4 h in medium supplemented with ascorbate. After exchange of medium, the cells were washed and then treated

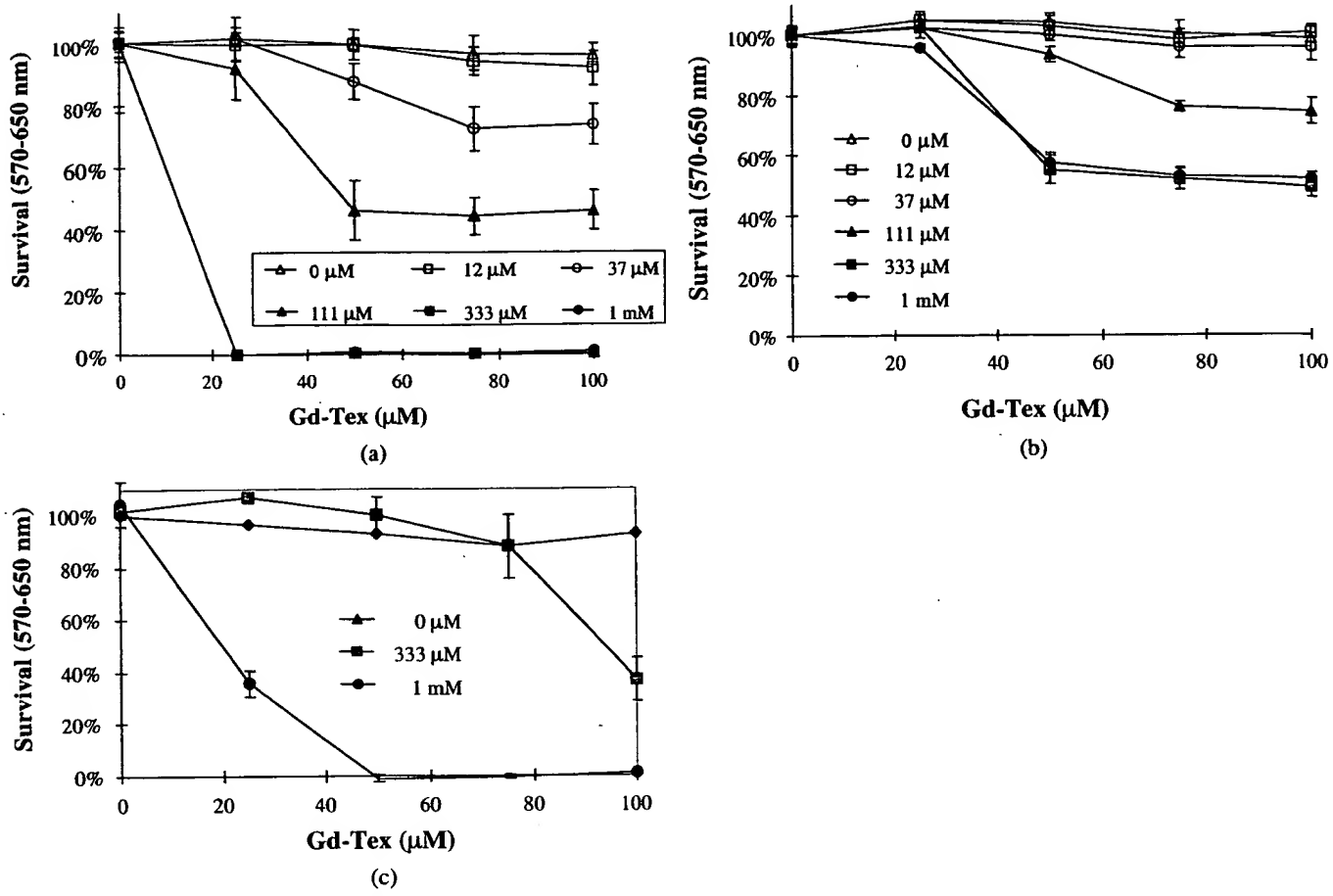

Fig. 4. Proliferation of MES-SA human uterine cancer cells in the presence of Gd-Tex and (a) ascorbate, (b) ascorbate with superoxide dismutase and catalase, or (c) NADPH. Ascorbate or NADPH alone had no effect at the concentrations displayed. Legends refer to concentration of ascorbate or NADPH. Error bars indicate standard deviation (n = 4).

with a solution of DCFA in phosphate-buffered saline overnight. As shown in Fig. 5, a dose-dependent increase in fluorescence was observed in the cultures that were treated with Gd-Tex, indicative of DCFA oxidation and concomitant formation of dichlorofluorescein (24). The magnitude of this fluorescent signal was also found to vary in accordance with the concentration of ascorbate present in the medium during the incubation.

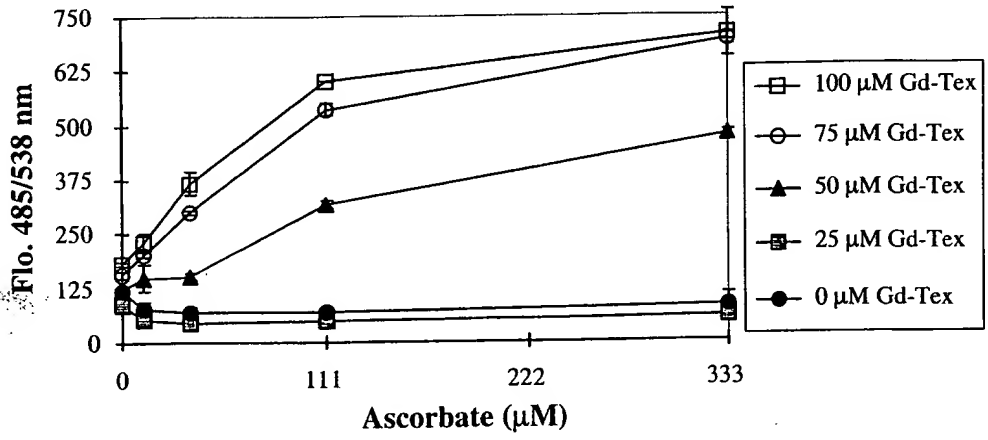

Fig. 5. Dichlorofluorescein fluorescence in MES-SA cell cultures (~2 × 10^4 cells/well) treated with Gd-Tex and ascorbate for 4 h. The fluorescence (arbitrary units) was measured after 18 h using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. No cytotoxicity as measured by MTT on parallel cultures was observed at the ascorbate concentrations shown. Error bars indicate standard deviation (n = 2).

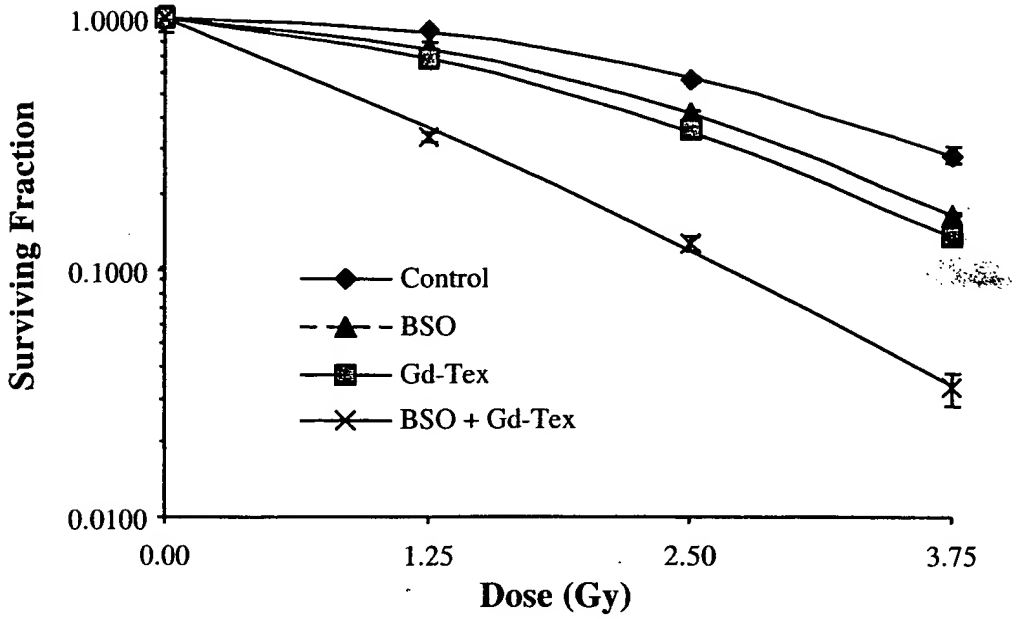

Fig. 6. Radiation response measured by clonogenic assay of MES-SA human uterine cancer cells after treatment with Gd-Tex or BSO. BSO (100 μM final concentration) and Gd-Tex (50 μM) were added 24 h before irradiation under ambient conditions. Glutathione levels were lowered to approximately 30% of controls by BSO treatment. Error bars indicate standard error (n = 3).

Radiation response

The effect of Gd-Tex on the response of MES-SA cells to ionizing radiation was studied by measuring clonogenic survival. Treatment of MES-SA cells with 50 μM Gd-Tex for 24 h before irradiation had a small but significant effect on radiation response, as did incubation with 100 μM BSO (Fig. 6). However, a considerable decrease in surviving fraction was observed in cells preincubated with both BSO and Gd-Tex. A similar degree of sensitization was observed whether the cells were treated with Gd-Tex for 24 h before or after exposure to ionizing radiation (data not shown). The radiation re-

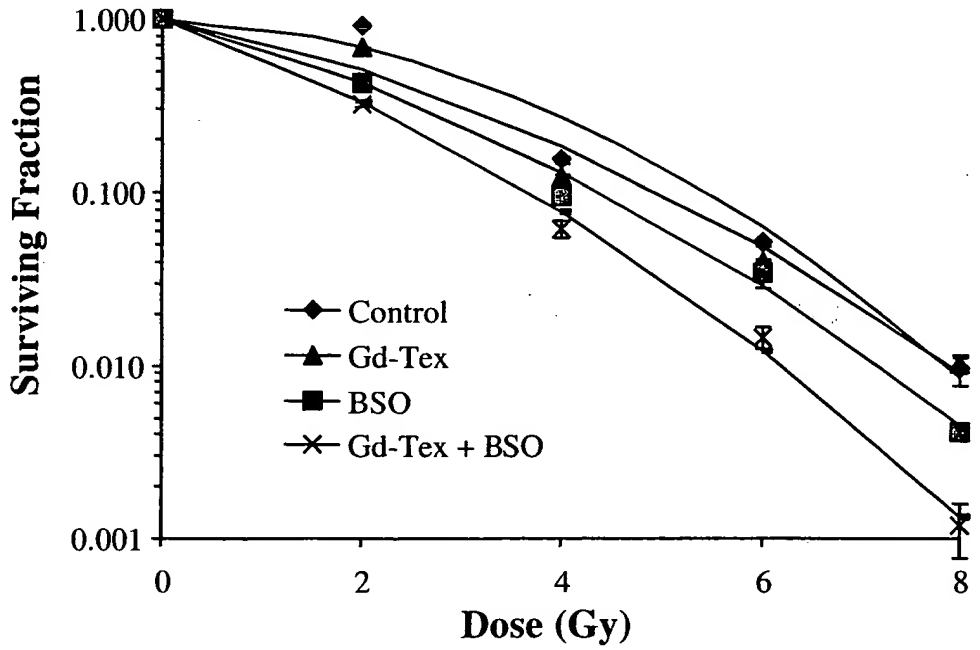

Fig. 7. Radiation response measured by clonogenic assay of A549 human lung cancer cells after treatment with Gd-Tex or BSO. Treatment conditions were identical to those used in Fig. 6. Error bars indicate standard error (n = 4).

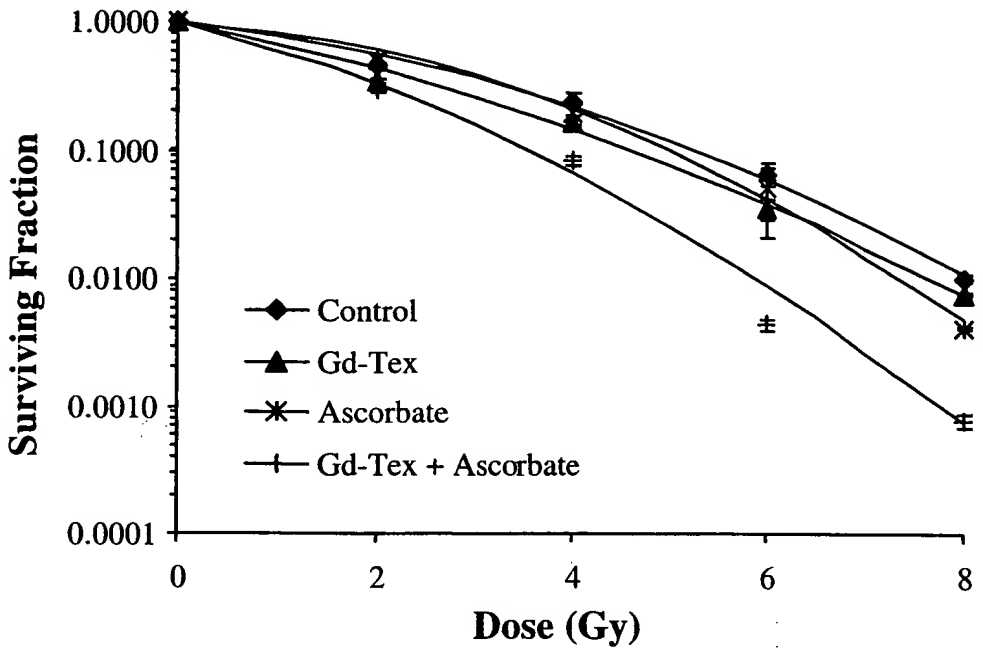

Fig. 8. Radiation response measured by clonogenic assay of E89 CHO cells after treatment with Gd-Tex. Gd-Tex (25 μM) and ascorbate (37.5 μM) were added 3 h before irradiation under ambient conditions. Error bars indicate standard error (n = 3).

sponse in A549 cultures was also enhanced by treatment with BSO and Gd-Tex (Fig. 7).

The CHO cell line variant E89 is deficient in the pentose cycle enzyme glucose-6-phosphate dehydrogenase (20). Incubation with Gd-Tex led to greater growth inhibition in this line, as compared to the wild-type cell line K1, even in the absence of exogenous ascorbate (e.g., surviving fraction <1% in E89 after a 29-h treatment with 100 μM Gd-Tex vs. 60% in K1, MTT data). Treatment of E89 cells with 25 μM Gd-Tex for 3 h before irradiation had no effect on the radiation response in the absence of ascorbate, but significantly lowered the surviving fraction where exogenous ascorbate (37.5 μM) was present (Fig. 8).

To ascertain whether these treatment conditions lead to changes in cellular levels of reduced thiols, nonprotein thiol and protein thiol fractions were assayed in plateau phase cultures using DTNB. After E89 cells were treated for 3 h with 50 μM Gd-Tex and 3.75 mM ascorbate (ascorbate concentration was adjusted to match cell number), levels of both protein and nonprotein thiols were found to decrease, by 11($\pm$1.2)% and 31($\pm$4.2)%, respectively. Similar decreases in cellular levels of reduced thiols have not been detected, thus far, in other cell lines tested (e.g., A549), presumably because of the greater capacity of these lines to reduce oxidized thiols (cf. "Discussion").

The murine B-cell lines LYAR and LYAS have been shown to be resistant or sensitive, respectively, to apoptosis induced by ionizing radiation (21). Exposure of these cells to 50 μM Gd-Tex for 18 h before radiation led to significant sensitization in LYAS, but not in LYAR (Fig. 9). Further study revealed that the level of sensitization observed did not correlate with the amount of ascorbate present in the medium at the onset of incubation (data not shown).

DISCUSSION

Co-incubation of NADPH (or NADH) with catalytic amounts of Gd-Tex rapidly converted this cofactor to its oxidized form in buffer (Fig. 2). This led to the production of hydrogen peroxide under aerobic conditions (Fig. 3). Similarly, ascorbic acid can serve as a source of reducing equivalents, to form superoxide and, ultimately, hydrogen peroxide (Fig. 3). Such "futile" redox cycling has frequently been encountered as a property of electron-affinic molecules (25). The specific rate at which this can occur depends in part, for texaphyrin metal cation complexes, on the affinity for a particular substrate (cf. Fig. 2). The observed interactions with ascorbate and NADPH are consistent with the characterization of lanthanide cations as "hard" or "oxophilic," and with the open sites for axial coordination provided by the texaphyrin macrocyclic system. Interestingly, under inert atmosphere, the characteristic Gd-Tex absorbances were observed to bleach in the presence of NADPH. No reaction was observed in the presence of ascorbate under inert atmosphere, presumably because of the less favorable potential for this redox couple. These observations raise the question whether irreversible loss of drug might occur in regions of tumors that are sufficiently hypoxic. Whether such a process occurs *in vivo*, and what effect, if any, released gadolinium cation would have on cells in such a circumstance, is at present unknown.

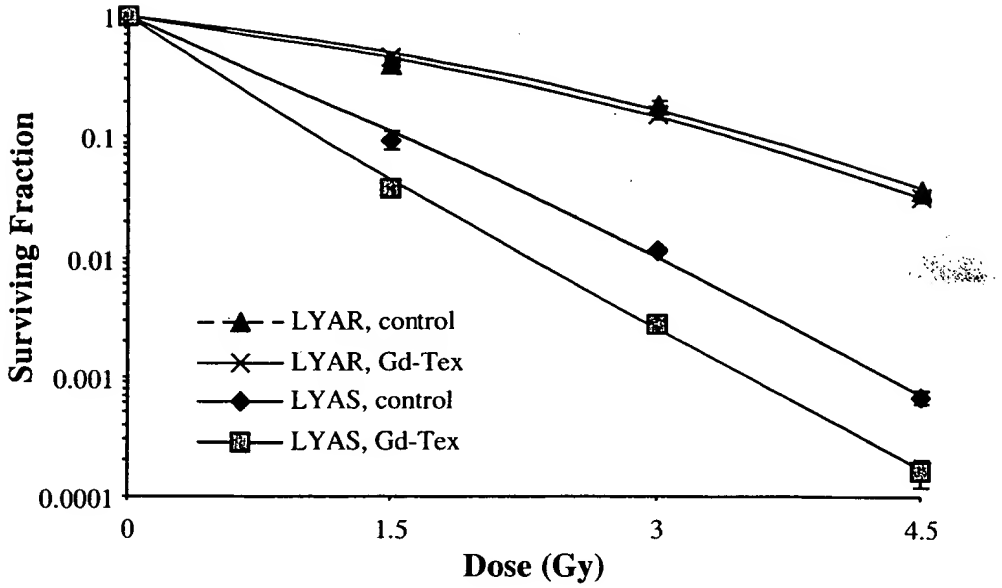

Fig. 9. Radiation response measured by clonogenic assay of murine lymphoma cells LYAR and LYAS after treatment with Gd-Tex. Gd-Tex (50 μM) was added 17 h before irradiation under ambient conditions. Error bars indicate standard error (n = 3).

The catalytic properties of Gd-Tex observed in buffer were retained under cell culture conditions (Fig. 4). For example, incubation with Gd-Tex and ascorbate led to greater cell growth inhibition than the NADPH/Gd-Tex combination. The presence of catalase, together with superoxide dismutase, afforded partial cellular protection (Fig. 4b). By removal of superoxide, superoxide dismutase is expected to inhibit its reduction by ascorbate, thereby slowing overall ascorbate oxidation. Catalase alone also afforded cellular protection (data not shown). These data attest to the formation of superoxide and hydrogen peroxide under cell culture conditions. It is important to note that the cellular inhibition shown in Fig. 4 may be a consequence of, primarily, extracellular oxidation of ascorbate or NADPH. One would expect the degree of this inhibition to be progressively reduced at higher cell numbers, commensurate with the capacity of cells to inactivate hydrogen peroxide via the action of enzymes such as catalase and glutathione peroxidase. Nonetheless, these findings are consistent with the idea that related processes can occur intracellularly. For example, DCFA oxidation was increased in a manner that depended on the concentration of both Gd-Tex and ascorbate (Fig. 5). DCFA is an intracellular probe for reactive oxygen species such as superoxide and hydrogen peroxide (24). Moreover, incubation of cells with Gd-Tex and BSO, diamide, or antimycin A leads to a cooperative decrease in cellular proliferation in the absence of exogenous reducing metabolites (26). Antimycin A blocks semiubiquinone reduction at mitochondrial complex III, leading to intracellular superoxide formation (27). BSO inhibits the biosynthesis of glutathione and can lead to a decrease of glutathione peroxidase activity (28). Diamide rapidly oxidizes glutathione and other protein thiols stoichiometrically (2, 3). Considered in concert, these data provide strong support for the conclusion that Gd-Tex treatment leads to oxidative stress, consistent with intracellular redox cycling.

Exposure to either *tert*-butyl hydroperoxide or hydrogen peroxide (produced during radiolysis) has been reported to enhance the radiation response of cells depleted of glutathione using BSO (5, 6). The cooperative effects of BSO and Gd-Tex seen in culture led us to consider whether these agents affect a common biochemical pathway and whether coadministration of BSO would potentiate the radiation-sensitizing properties of Gd-Tex. Indeed, we found that treatment with Gd-Tex led to an enhanced radiation response in MES-SA cells and A549 cells depleted of glutathione as a result of treatment with BSO (Figs. 6 and 7). Interestingly, we observed sensitization to a similar degree when the MES-SA cultures were treated with Gd-Tex immediately after irradiation (data not shown). This latter finding is consistent with involvement of a downstream process such as DNA repair. A perhaps related possibility is that inhibition of glutathione peroxidase (by glutathione depletion), coupled with hydrogen peroxide formation, might lead to DNA damage independent of that produced by irradiation (29).

We have also considered whether Gd-Tex treatment could affect glutathione status. We found that reduced glutathione was directly oxidized by Gd-Tex in buffer, albeit at an approximately 42-fold lower rate than ascorbate (based on the rate of oxygen consumption at 1.25 mM substrate concentration) (26). However, redox cycling by Gd-Tex could promote the oxidation of glutathione indirectly, because of the interconversion of cellular antioxidants. For example, as outlined in Fig. 10, superoxide would be formed after ascorbate oxidation by Gd-Tex. Further reduction of superoxide by ascorbate, or disproportionation of this species by superoxide dismutase, would lead to hydro-

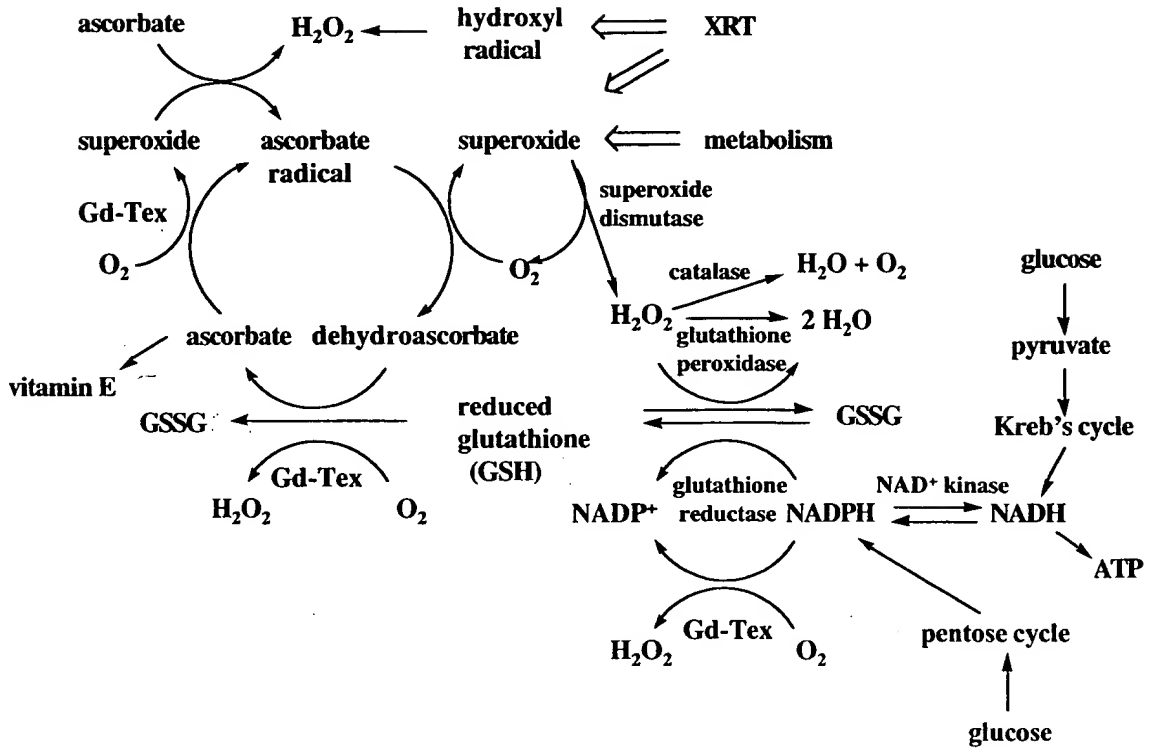

Fig. 10. Cellular antioxidant system showing relationship of ascorbate, glutathione, and NADPH. Positions of proposed Gd-Tex interaction are indicated. Reactive oxygen species derived from radiolysis of water (XRT) or metabolism are also shown.

gen peroxide formation. Ionizing radiation can also lead to hydrogen peroxide, via hydroxyl radical combination. Enzymatic reduction of hydrogen peroxide, in turn, consumes reduced glutathione, as does regeneration of ascorbate from dehydroascorbate (The latter is formed from ascorbate radical either by disproportionation or electron transfer to oxygen). NADPH, which is required to regenerate reduced glutathione, may also serve as a substrate for texaphyrin-mediated redox cycling (see above). However, both glutathione and NADPH would be maintained in their reduced forms via the action of the pentose cycle, the major source of cellular NADPH.

Considerations such as those outlined above suggested to us that the effects of redox cycling by Gd-Tex might be masked in cell culture by normal metabolism. For example, the pentose cycle was reported to be stimulated after treatment with *tert*-butyl hydroperoxide, diamide, or a redox cycling agent, methylene blue (30). Conversely, CHO cell lines deficient in glucose-6-phosphate dehydrogenase activity were found to be more susceptible to *tert*-butyl hydroperoxide, diamide, and (in at least one cell line, E48) ionizing radiation (31). When we incubated the glucose-6-phosphate dehydrogenase–deficient line E89 with Gd-Tex, we found the drug was more cytotoxic than in the parent K1 line. Moreover, the radiation response of this cell line was enhanced when treated with Gd-Tex and ascorbate for 3 h (Fig. 8). Measurement of reduced thiol levels in this cell line confirmed that protein and nonprotein thiols were depleted by 11% and 31%, respectively, presumably as a consequence of hydrogen peroxide formation. Depletion of reducing metabolites in E89 by disulfide treatment has been reported to enhance radiation response via DNA repair inhibition (32). A similar mechanism may be invoked to explain the sensitizing effect of Gd-Tex and ascorbate, highlighting the importance of the pentose cycle in maintaining damage repair under conditions of oxidative stress.

We also observed radiation enhancement in the apoptosis-sensitive B-cell lymphoma LYAS, but not in the corresponding apoptosis-resistant line LYAR (Fig. 9). It seems likely that the radiation enhancement observed in the presence of Gd-Tex is again a consequence of oxidative stress due to redox cycling. Oxidative stress can induce mitochondrial permeability transition pore opening, a key event in apoptosis (33). Enhanced apoptosis has recently been reported to occur where superoxide dismutase activity was inhibited either pharmacologically or by antisense expression in leukemia cells and in colon cancer cell lines, respectively (34, 35). These findings, which appear to be limited to cell lines in possession of intact apoptotic capability, support the notion that increasing intracellular levels of superoxide can lead to enhanced radiation response.

The findings presented here provide an explanation for the cell culture data reported by Bernhard *et al.* (36). Our present data lead us to suggest that the outcome of *in vitro* experiments with Gd-Tex can be variable, depending on the cell line and media conditions. For example, we find modest

radiation enhancement with Gd-Tex in the A549 cell line when the cells are treated with BSO. Bernhard *et al.*, on the other hand, failed to show radiation enhancement with A549 in the absence of BSO (36). Our present findings lead us to conclude that cell-line–dependent differences in metabolism or reducing metabolite levels can alter the degree to which sensitization by Gd-Tex is observed *in vitro*. Media employed for these experiments typically contain 1–5 g/L glucose, often 1 mM pyruvate, and no ascorbate. Given the importance of the pentose cycle in replenishing reducing metabolites (Cf. Fig. 10), it would not be surprising to discover that the effect of redox cycling could be masked under cell culture conditions. Considering the indirect, non-"oxygen mimetic" mechanism of action we propose for Gd-Tex, it is our belief that the *in vitro* system must be chosen with care to model accurately the effect of the drug *in vivo*.

Cellular levels of ascorbate in humans typically range from 200 to 800 μM, whereas plasma levels are lower, approximately 25–50 μM (29, 37, 38). Interestingly, tumor levels of ascorbate and other reducing metabolites have been found to be elevated, for example, in human breast cancer (39). Texaphyrins can also display considerable (greater than 10-fold) selective accumulation in tumor tissue. This selective biolocalization, which is considered integral to the pharmaceutical development of the compounds, has been demonstrated using magnetic resonance imaging, radioactive labeling, and fluorescent imaging techniques (13–17, 40–46). Recently, tumor energy metabolism has been shown by ^{31}P NMR to be compromised after Gd-Tex injection in tumor-bearing animals (47). This finding, coupled with the results of the present study, provides support for the notion that redox cycling by Gd-Tex contributes to the increased radiation response observed in animal models (14, 17, 48).

Recent data demonstrate that Gd-Tex increases tumor cell cytotoxic response to certain chemotherapeutic agents, such as bleomycin and doxorubicin (49). These results are consistent with the mechanistic explanations presented here. Although not completely understood, the effects of Gd-Tex redox cycling could be substantial *in vivo*, where nutritional deprivation, reperfusion, and other factors could render cells more susceptible to oxidative stress. Our findings suggest that it may be possible to use texaphyrins to exploit these differences and develop improved treatments for cancer and other diseases.

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